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Respectfully submitted,

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# Inhibitors of the Anandamide Transporter as Analgesic Agents

## Government Funding

This invention was made with Government support under Contract No. DA 3801 awarded by the National Institute of Drug Abuse. The Government has certain rights in the invention.

## Related Application

This application is based on and claims the benefit under 35 U.S.C. §119(e) of United States Provisional Application No. 60/088,568 filed June 9, 1998.

## Background of the Invention

The marijuana derived cannabinoid $\Delta^9$-tetrahydrocannabinol, $\Delta^9$THC, is known to bind to CB1 receptors in the brain and CB2 receptors in the spleen. Compounds which stimulate those receptors have been shown to induce analgesia and sedation, to cause mood elevation including euphoria and dream states, to control nausea and appetite and to lower intraocular pressure. Cannabinoids have also been shown to suppress the immune system. Thus, compounds which stimulate the receptors, directly or indirectly, are potentially useful in treating glaucoma, preventing tissue rejection in organ transplant patients, controlling nausea in patients undergoing chemotherapy, controlling pain and enhancing the appetite and controlling pain in individuals with AIDS Wasting Syndrome.

In addition to acting at the receptors, cannabinoids also affect cellular membranes, thereby producing undesirable side effects such as drowsiness, impairment of monoamine oxidase function and impairment of non-receptor mediated brain function. The addictive and

psychotropic properties of cannabinoids also limit their therapeutic value.

Arachidonyl ethanolamide (anandamide) is an endogenous lipid that binds to and activates cannabinoid receptors and mimics the pharmacological activity of $\Delta^9$THC. In general, anandamide has been found to be somewhat less potent than $\Delta^9$THC. Despite having a rapid onset of action, the magnitude and duration of action of anandamide is relatively short, presumably because of a rapid inactivation process consisting of carrier-mediated transport into cells followed by intracellular hydrolysis by a membrane-bound amidohydrolase, anandamide amidase. Thus, inhibitors of anandamide amidase have the effect of indirectly stimulating the receptors by increasing *in vivo* levels of anandamide. In this connection, attention is directed to Makriyannis et al U.S. Patents 5,688,825 and 5,874,459, the disclosures of which are incorporated herein by reference.

Anandamide released by depolarized neurons is believed to be subject to rapid cellular uptake followed by enzymatic degradation. Indeed, rat brain neurons and astrocytes in primary culture avidly take up radioactively labeled anandamide through a mechanism that meets four key criteria of a carrier-mediated transport; temperature dependence, high affinity, substrate selectivity, and saturation. In that other lipids including polyunsaturated fatty acids and prostaglandin $E_2$ ($PGE_2$) enter cells by carrier-mediated transport, it is possible that anandamide uses a similar mechanism. This accumulation may result from the activity of a transmembrane carrier or transporter, which may thus participate in termination of the biological actions of anandamide. This carrier or anandamide transporter is believed to be involved in the inactivation of anandamide. Thus, anandamide released from neurons on depolarization may be rapidly transported back into the cells and subsequently hydrolyzed by an amidase thereby terminating its

biological actions. Consequently, the anandamide transporter is a potential therapeutic target for the development of useful medications.

There is considerable interest in understanding the mechanism of anandamide transport and in developing pharmacological agents that selectively interfere with it. Anandamide transport inhibitors may be used as experimental tools to reveal the possible physiological functions of this biologically active lipid. Many of these functions are still elusive despite a growing body of evidence suggesting that the endocannabinoid system is intrinsically active not only in brain and spinal cord, but also in peripheral tissue. Furthermore, anandamide transport inhibitors may offer a rational therapeutic approach to a variety of disease states, including pain, psychomotor disorders, and multiple sclerosis, in which elevation of native anandamide levels may bring about a more favorable response and fewer side effects than direct activation of CB1 receptors by agonist drugs.

**Summary of the Invention**

It has now been found that certain analogs of anandamide are potent inhibitors of transport of anandamide across cell membranes. The transport inhibitor does not activate the cannabinoid receptors or inhibit anandamide hydrolysis *per se* but instead prevents anandamide reuptake thereby prolonging the level of the undegraded anandamide. Previously, cannabinoid drugs were targeted toward cannabiniod receptors and amidase enzymes. The anandamide transport inhibitor of the present invention targets activity of the anandamide transporter.

The inhibitors are analogs of anandamide and exhibit the tail, central and head pharmacophore portions represented by Structural Formula I

X - Y - Z (I)

wherein tail portion X is a fatty acid chain remnant or a biphenyl group with an akyl chain, central portion Y is a member selected from the group consisting of -NA-C(O)-, -NH-, -NH-C(O)-NH-, -NH-C(O)-O-, -C(O)-NH, -O-C(O)-, -O-, -S- and -H and head portion Z is selected form the group consisting of hydrogen, aryl, alkyl aryl, halogen substituted alkyl aryl, cyclic glycerols and substituted cyclic glycerols.

The novel inhibitors of the present invention, when tested *in vitro*, inhibit accumulation of anandamide in rat cortical neurons and astrocytes and enhance various effects of anandamide administration both *in vitro* and *in vivo*. The vasodepressor responses are significantly potentiated and prolonged by the transport inhibitors. Thus, the inhibitors are believed to be effective drugs for the treatment of cardivascular diseases and blood pressure disorders.

The novel biochemical pathway involving the anandamide transporter system and composition disclosed herein have other therapeutic uses. For example, the compounds and methods of the present invention, like cannabinoids, can be effective in the relief of the pain caused by cancer and the nausea resulting from cancer chemotherapy as well as for the relief of peripheral pain. Beneficially, they would not be expected to have the undesirable membrane-related side-effects associated with cannabinoids. In addition, the methods and compounds disclosed herein may be immunosuppressive and can therefore be used to prevent organ rejection in an individual undergoing an organ transplant. Because the compounds and methods of the present invention enhance the appetite of an individual, they can be used to treat patients with AIDS Wasting Syndrome, who are often suffering from malnourishment as a result of appetite loss. The compounds could also be used in psychomotor disorders and multiple sclerosis and peripheral hypertension. In all of the above conditions, evaluation of anandamide levels may bring about a more favorable

response and fewer side effects than direct activation of CB-1 and CB-2 receptors by agonist drugs.

The novel inhibitors of anandamide transport disclosed herein also have research uses. For example, they can be used to maintain the level of anandamide *in vivo* to study the effect of anandamide on individuals and animals. The anandamide transport inhibitors disclosed herein can also be used as an aid in drug design, for example as a control in assays for testing other compounds for their ability to inhibit anandamide transport and to determine the structural and activity requirements of such inhibitors. These results, together with data from initial experiments on the selectivity of radioactively labeled [$^3$H]anandamide uptake by rat brain astrocytes, suggest that the interactions of anandamide with its putative transporter protein are governed by strict structural requirements. These results delineate the broad molecular requisites for this process, thus providing a basis for the design of more potent and selective inhibitors with potential applications to medicine.

Anandamide uptake in neurons and astrocytes has been found to be mediated by a high-affinity, $Na^+$-independent transporter that is selectively inhibited by the inhibitors of the present invention. The structural determinants governing recognition and translocation of substrates by the anandamide transporter have been determined. The secondary amido group interacts favorably with the transporter, but may be replaced with other radicals, suggesting that it may serve as hydrogen acceptor. Putative endogenous cannabinoid esters also serve as a substrate for the transporter. Substrate recognition and translocation require the presence of at least one cis double bond situated at the middle region of the fatty acid hydrocarbon chain or a biphenyl group with an aliphatic chain, indicating a preference for ligands whose hydrophobic tail can adopt a bent U-shaped or hair-pin

configuration. Uptake experiments with radioactively labeled substrates favor two or more and preferably four cis nonconjugated double bonds for optimal translocation across the cell membrane, suggesting that substrates are transported in a folded hairpin conformation.

**Brief Description of the Figures**

Fig. 1 is a graph showing the translocation of substrate inhibitors of the present invention at different concentration levels.

Fig. 2 is a graph similar to Fig. 1 for two different substrate inhibitors.

**Detailed Description of the Invention**

One embodiment of the present invention is directed to the discovery of a putative anandamide transporter system which has been characterized biochemically and pharmacologically and which can be used as a target for the discovery of novel medications. These would include all compounds that can inhibit the function of this transporter. The invention further includes the pharmacological formula containing an effective amount of the inhibitor while another embodiment is directed to a method of inhibiting anandamide transport in an individual or animal by administering a therapeutically effective amount of the inhibitor and/or physiologically acceptable salts thereof. The inhibition results in increased levels of anandamide in the individual or animal, thereby causing prolonged stimulation of cannabinoid receptors in the individual or animal, e.g., the CB1 receptor in the brain and the CB2 receptor in the spleen. Thus, the present invention involves not only the inhibitor itself but also a method of reducing anandamide transporter activity in an individual or animal. It is to be understood that the present invention may also be used to reduce the activity of transporters

not yet discovered for which anandamide and/or a cannabinoid act as an agonist.

The anandamide transport inhibitors of the present invention are amide, reverse amide or carbonyl amine, urea, carbamate and ester analogs of anandamide having the three pharmacophores of the Structural Formula I wherein the tail portion X is a fatty acid hydrophobic carbon chain having one or more nonconjugated cis double bonds in the middle portion of the aliphatic hydrocarbon chain or a biphenyl group having an alkyl or branched alkyl distal moiety of about 1 to about 10 carbon atoms. The biphenyl group is substituted with 1 - 6 substituents including OH, $CH_3$, halogen, $SCH_3$, $NH_2$, NHCOR, $SO_2NHR$, $NO_2$ The fatty acid chain may contain four to thirty carbon atoms but preferably the chain length is about 10 to 28 carbon atoms and more preferably contains from about 17 to about 22 carbon atoms. The aliphatic hydrocarbon chain may terminate with an aryl or alkyl aryl group. By contrast, analogs with fully saturated chains or with a trans or terminal double bond fail to compete successfully with [$^3$H]anandamide for transport and thus are ineffective as inhibitors. The central pharmacophore Y is selected from the group set forth hereinbefore. However, compounds containing a free carboxylic acid, carboxyethyl and carboxymethyl groups, or a primary alcohol are inactive. The head portion Z is selected from the group set forth hereinbefore.

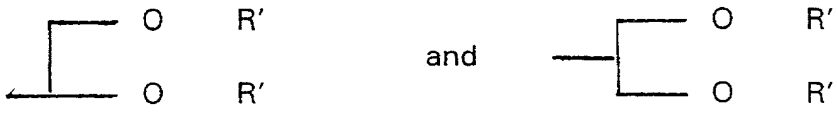
As used herein, "aliphatic hydrocarbon" includes, unless otherwise stated, one or more polyalkylene groups connected by one or more *cis*-alkenyl linkages such that the total number of methylene carbon atoms is within the ranges set forth herein. The structure of preferred tail portions have the formula II

$$CR_3\text{-}(CR_2)_a\text{-}(cis\text{-}CH=CHCrR_2)_b\text{-}(CR_2)_c\text{-} \qquad (II)$$

wherein R is selected from the groups consisting of hydrogen and lower alkyl groups, however the chain's terminal R may include phenyl and biphenyl groups that are unsubstituted or substituted with a member selected form the group consiting of hydroxyl, halogen, $-NO_2$, $-NH_2$, $-SCH_3$, $-CH_3$ and $-OCH_3$ and a and c are integers 0 and 1 through 10 and b is an integer from 1 through 6. Specific examples include structures where X is $CH_3$ - $(CH_2)_4$ - (*cis*-$CH=CHCH_2$-)$_4$ - $(CH_2)_2$-, $CH_3$-$(CH_2)_4$- (*cis*-$CH=CHCH_2)_3$-$(CH_2)_5$, - $CH_3$ - $(CH_2)_6$ - (*cis*-$CH=CHCH_2)_2$ - $(CH_2)_6$ -, $CH_3$ - $(CH_2)_6$ - (*cis*-$CH=CHCH_2)_2$ - $(CH_2)_5$ - $CH_3$-$(CH_2)_7$-*cis* $CH=CH$-$(CH_2)_9$, $CH_3$ - $(CH_2)_7$ - *cis* - $CH=CH$-$(CH_2)_7$ - and $CH_3$-$(CH_2)_4$-$(CH=CHCH_2)_4$-$CH_2$-$C(CH_3)_2$- A lower alkyl group is a straight or branched chain alkyl group having 1 to 5 carbon atoms, unless otherwise stated.

As used herein, an "aryl" group is a carbocyclic aromatic ring system such as phenyl, biphenyl 1-naphthyl or 2-naphthyl.

As used herein "cyclic glycerols" include members selected from the group consisting of



wherein R' is a member selected form the group consisting of hydrogen, lower alkyl, aryl and substituted aryl radicals.

Typical procedures for synthesizing these materials are as follows:

**Arachidonyl alcohol:** To a magnetically stirred solution of 0.5 ml (0.5 mmol) of $LiAlH_4$ in $Et_2O$, 100 mg (0.314 mmol) of arachidonic acid methyl ester in 2 mL of $Et_2O$ was added dropwise at 0°C. The reaction mixture was stirred for 1 h and then quenched by addition of 1 mL of EtOAC. 2 mL of saturated $NH_4Cl$ solution was added and the organic layer was separated, dried with $MgSO_4$, filtered and evaporated. Chromatography on silica gel (eluents: $CH_2Cl_2$/petroleum ether up to 70% $CH_2Cl_2$), evaporation, folowed by millipore filtration of a $CH_2Cl_2$

solution of the product gave 99.3 mg (0.292 mmol, 93% yield) of arachidonyl alcohol as a colorless oil: TLC ($CHCl_3$) $R_f$ 0.28; $^1H$ NMR (200 MHz, $CDCl_3$) δ 5.37 (m, 8 H ), 3.61 (t, 2 H, $J$ = 6 Hz), 2.79 (m, 6H), 2.08 (m, 4 H), 1.66 - 1.17 (m, 8 H), 0.92 (t, 3 H, $J$ = 7 Hz); Anal. C, H.

**Arachidonyl azide:** To a magnetically stirred solution of 50 mg (0.17 mmol) of arachidonyl alcohol in 1 mL of pyridine 29.2 mg.(0.255 mmol) of mesyl chloride was added at 0°C. After stirring for 5 h, the reaction mixture was poured into 2 mL of iced water and extracted with $Et_2O$ (2 x 4 mL). The ethereal layers were combined and washed with 1 N $H_2SO_4$, $NaHCO_3$, and evaporated *in vacuo* to dryness. The mesylate was not purified and it was directly converted to the corresponding azide: it was dissolved first in 2 ml DMF and then 4 ml of solution of 6.5 mg. (0.85 mmol) $NaN_3$ in DMF was added at room temperature. The reaction mixture was heated at 90°C for 24 h. After the reaction mixture was cooled down to room temperature the inorganic material was filtered off and the filtrated was poured into 1 mL of iced $H_2O$ and then extracted with $Et_2O$ (2 x 6 mL). The etheral layers were combined, dried, filtered, and evaporated *in vacuo* to dryness. Silica gel chromatography (eluent: petroleum ether), evaporation, followed by millipore filtration of a $CH_2Cl_2$ solution of the product gave 39 mg (0.12 mmol, 73% yield) of arachidonyl axide as a colorless oil: $^1H$ NMR (200 MHz, $CDCl_3$) δ 5.38 (m, 8 H), 3.27 (t, 2 H, $J$ = 6 Hz), 2.81 (m, 6H), 2.11 - 2.01 (m, 4), 1.62 (m, 2 H), 1.48 - 1.25 (m, 6 H), 0.89 (t, 3 H, $J$ = 7 Hz). Ana. C, H, N.

**Arachidonylamine:** To a magnetically stirred solution of 132 mg (0.43 mmol) of arachidonyl azide in 3 mL of $Et_2O$, 4 mL of a 1.0 M LAH solution in THF (4.0 mmol) was added dropwise at room temperature. The reaction mixture was refluxed for 3 h and then it was cooled to ambient temperature. 210 mg (5mmol) of NaF was added and the

reaction was quenched with wet $Et_2O$. The white mixture was filtered and the solvent was evaporated to dryness. Silica gel chromatography (eluents: $CH_2Cl_2$/MeOP-up to 50% MeOH), evaporation of solvent, followed by millipore filtration of a $CH_2Cl_2$ solution of the product then gave 78.9mg (0.28 mmol, 64% yield) of arachidonyl-amine as a colorless oil. TLC (EtOAc/$CH_2Cl_2$ (20:80)) $R_f$ 0.33; $^1H$ NMR (200 MHz, $CDCl_3$) δ 5.38 (m, 8 H), 2.82 (m, 6 H), 2.70 (t, 2 H, $J$ = 6.6 Hz), 2.08 (m, 4 H), 1.40 (m, 4 H), 1.26 (m, 6 H), 0.89 (t, 3 H, $J$ = 6.4 Hz).

**Arachidonylamine-3'-(hydroxy)-propionate:** To a magnetically stirred solution of 48 mg (0.17 mmol) of arachidonyl-amine in 2 mL of $CH_2Cl_2$, 58 μl (0.17 mmol) of a 2.0 M solution of $(CH_3)_3Al$ in of hexane were added at room temperature. The mixture was stirred for 20 min and then 12.24 mg (0.17 mmol) of β-propiolactone was added dropwise. The reaction mixture was refluxed for 6 h, quenched with 1N Hcl and extracted with methyl chloride. The product was purified with silica gel column chromatography (eluents with $CH_2Cl_2$/EtOAc, up to 80% EtOAc. Evaporation of the solvent, followed by millipore filtration of a $CH_2Cl_2$ solution of the product gave 51 mg ( 0.14 mmol, 83% yield) of arachidonyl-amine-3'-(hydroxy)-propionate as a colorless oil; TLC (EtOAc) $R_f$ 0.26; $^1H$ NMR (200 MHz, $CDCl_3$) δ 5.35 (m, 8 H), 3.85 (q, 2 H, $J$ = 5.4 Hz), 3.25 (q, 2 H,J = 5.4 Hz), 2.84 (m, 6 H), 2.66 (t, 2 H, $J$ = 6.8 Hz), 2.05 (m, 4 H), 1.57 (m, 2 H), 1.35 (m, 6 H) 0.89 (t, 3, H, $J$ = 6.5 Hz); Anal. C, H, N.

**Arachidonyl-amine-trifluoroacetate:** To a magnetically stirred solution of 69 mg (0.6 mmol) of trifluoro acetic acid, in 2ml of dry methylene chloride, at 0°C, 0.046 ml (0.6 mmol) of dry DMF was added and then 0.3 ml (0.6 mmol) of 2.0 M solution of oxalyl chloride in methylene chloride, dropwise. The reaction mixture was stirred for 20 mins and then a solution of 172 mg (0.6 mmol) of arachidonyl amine in 2 ml of methylene chloride was added and the reaction was stirred for 2 hrs at

ambient temperature. The product was purified with silica gel column chromatography (eluents: petroleum ether/ethyl acetate, up to 50% ethyl acetate). Evaporation of the solvent, followed by millipore filtration of a $CH_2Cl_2$ solution of the product gave 153 mg (0.4 mmol, 67% yield) of arachidonyl-amine-trifluoroacetate as a colorless oil.

Exploration of the Y and Z pharmacophores shows that compounds containing primary, secondary and tertiary amido groups as well as hydroxyethyl ester or glycerol ester moieties are capable of competing with [$^3$H]anandamide, but exhibit a wide range of potencies. Structural variations of the head group Z leads to analogs with diverse selectivities for the anandamide transporter. Thus substitution of the terminal hydroxyl with a hydrogen causes a substantial decrease in potency, whereas replacement of the entire hydroxyalkyl moiety with hydrogen yields compounds that are as potent as anandamide. Introduction of a methyl group alpha to the amido nitrogen also leads to active compounds. Chiral molecules display considerable enantioselective inhibition of [$^3$H]anandamide transport. The (*S*) enantiomer is approximately four times more potent than its (*R*) isomer.

The most striking structure-activity correlation was observed with analogs having hydroxyphenyl radicals at the head group. Use of the hydroxyphenyl group leads to relatively potent uptake inhibitors, with the 4-hydroxyphenyl analog being distinctly the most successful. Conversely, the 4-methylphenyl analog as well as other analogs with electron donating or electron withdrawing para substituents display no significant activity. Varying these substituents from the para to the meta or ortho position does not restore activity. Other analogs containing multiple substituents on the phenyl ring (e.g., 3-chloro-4-hydroxyphenyl) or a bulkier aromatic moiety [e.g., 1-(4-hydroxynaphthyl)] are also less potent than the 4-hydroxyphenyl group.

The transporter.

In order to properly evaluate the effectiveness of inhibitors of anandamide transport, it was necessary to establish the identity and character of the carrier-mediated transporter. The accumulation of radioactively labeled exogenous [$^3$H]anandamide by neurons and astrocytes fulfills several criteria of a carrier-mediated transport. It is a rapid process that reaches 50% of its maximum within about four minutes. Furthermore, [$^3$H]anandamide accumulation is temperature dependent and saturable. Kinetic analyses reveals that accumulation in neurons can be represented by two components of differing affinities (lower affinity: Michaelis constant, $K_m$ = 1.2 $\mu$M, maximum accumulation rate, $V_{max}$ = 90.9 pmol/min per milligram of protein; higher affinity: $K_m$ = 0.032 $\mu$M, $V_{max}$ = 5.9 pmol/min per milligram of protein). The higher affininy component may reflect a binding site, however, as it is displaced by the cannabinoid receptor antagonist, SR-141716-A (100 nM). In astrocytes, [$^3$H]anandamide accumulation is represented by a single high-affinity component ($K_m$ = 0.32 $\mu$M, $V_{max}$ = 171 pmol/min per milligram of protein). Such apparent $K_m$ values are similar to those of known neurotransmitter uptake systems and are suggestive therefore of high-affinity carrier-mediated transport.

To characterize further this putative anandamide transporter, cortical astrocytes in culture were employed. As expected from a selective process, the temperature-sensitive component of [$^3$H]anandamide accumulation was prevented by nonradioactive anandamide, but not by palmitoyl ethanolamide, arachidonate, prostanoids, or leukotrienes. Replacement of extracellular sodium ion with N-dimethylglocosamine or choline had no effect suggesting that accumulation is mediated by a $Na^+$- independent mechanism which has been observed for other lipids. Moreover, inhibition of fatty acid amide hydrolase (FAAH) activity indicates that an anandamide hydrolysis does

not provide the driving force for anandamide transport into astrocytes within the time frame of the experiment. Finally, the cannabinoid receptor agonist WIN-55212-2 (1 $\mu$M) and antagonist SR-141716-A (10 $\mu$M) also had no effect, suggesting that receptor internalization was not involved.

A primary criterion for defining carrier-mediated transport is pharmacological inhibition. To identify inhibitors of anandamide transport, examination was made of various components that prevent the cellular uptake of other lipids such as fatty acids, pholpholipids or bromcresol green. Among the compounds tested, only bromcresol green interfered with inanimate transport, albeit with limited potency and partial efficacy, bromcresol green inhibited [$^3$H]anandamide accumulation with an $IC_{50}$ (concentration needed to produce half-maximal inhibition) of 4 $\mu$M in neurons and 12 $\mu$M in astrocytes and acted noncompetitively. Moreover, bromcresol green had no significant effect on the binding of [$^3$H]WIN-55212-2 to rat cerebral membranes, on FAAH activity in brain microsomes and on uptake of [$^3$H]arachidonate or [$^3$H]ethanolamine in astrocytes.

The bromcresol green, which blocks $PGE_2$ transport, raised the question of whether anandamide accumulation occurred by means of a $PGE_2$ carrier. That this is not the case was shown by the lack of [$^3$H]$PGE_2$ accumulation in neurons or astrocytes and by the inability of $PGE_2$ to interfere with [$^3$H]anandamide accumulation. Previous results indicating that expression of $PGE_2$ transporter mRNA in brain tissue is not detectable further support this conclusion.

**[$^3$H] Anandamide competition assay using a high throughput method.**

Human CCF-STTG1 astrocytoma cells (American Type Culture Collection) were grown in RPMI 1640 culture medium containing 10%

FBS and 1 mM glutamine. Cells were seeded at a density of $2x10^5$/well = $6x10^5/cm^2$ and used at confluence (5 days post seeding). For standard competition assays, confluent cells grown in 96-well view plates were rinsed and preincubated for 10 min. at 37°C in Hanks Balanced Salt Solution (HBSS) supplemented to contain 138 mM NaCl, 5 mM Kcl, 1.26 mM MgSO4, 2.5 mM $CaCl_2$-$2H_2O$, 1mM phosphates, 4mM $NaHCO_3$, 10 mM glucose, 10 mM Hepes with 0.1% DMSO or 0.1% DMSO plus test compounds at their final concentrations (0.1 - 100 $\mu$M). Briefly, plates of cells were washed 3x with 100 ul with HBSS with 0.1% DMSO with a Multiwash Plus (Molecular Device) plate washer. Washed plates were placed into a plate warmer with an air:carbon dioxide mixture of 95:5.

A silanized 96 well plate was prepared as a mother plate for treating the cells. For each test compound a dilution sheet was generated to encompass a range of concentrations around a predicted $IC_{50}$ of 500 nM.

To the motherplate, 150 ul of a 2x dilution of test compound was added to two rows columns 1-12 or the 96 well mother plate. Add 150 ul of HBSS with 0.1% DMSO to each well of one of ROW A (label this row as *pretreatment*). To row B add 150 ul per well of [$^3$H]anandamide 100 or 1000 nM and label this row as *treatment*. This results in a 1x concentration of test compounds and a 50 or 500 nM concentration final concentration of anandamide.

Take the mother plate and set the electrapipette to fill 225 ul and dispense 50 ul of the pretreatment to the appropriate wells. Next decant the 96 well plate to remove the 100 ul of wash buffer. Add 50 ul per well for an n=4 columnwise down for rows a-d for test compound number 1. Then add 50 ul of compound 2 per well for 4 columnwise transfers to rows e-h. Place the plates back into the plate warmer/incubator.

After the 10 minute preincubation period, decant the plates. With the mother plate, set the electrapipette to fill 225 ul and dispense 50 ul of the treatment to the appropriate wells. Place the plate back into the plate warmer for 4 minutes. Then decant the plate into the hot sink and immediately aspirate the incubation media using the Filtermate 196 Cell Harvester (Packard Instruments, Meriden, CT), followed by rinsing the cells 6x with ice-cold HBSS containing 0.1% fatty acid free bovine serum albumin (Sigma).

Reactions were stopped by removing the incubation media and rinsing the cells three times with 0.1 ml of ice-cold HBSS containing 0.1% fatty acid-free BSA (Sigma). A final wash of the plate in HBSS was performed to remove any traces of albumin for the following protein analysis.

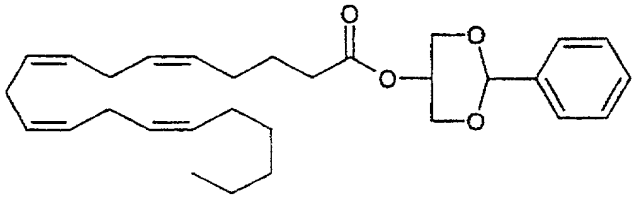
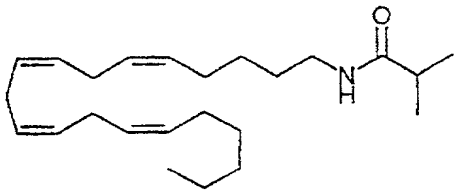
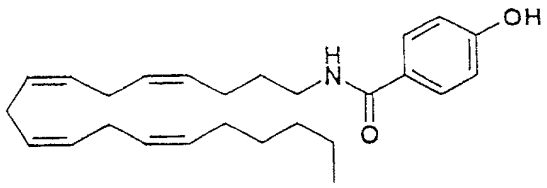
Cells were then solubilized by the addition of 50 ul/well of 1.2N NaOH/0.1% Triton X-100 and shaken on a plate shaker for 10 minutes. Aliquots of 15 ul were removed for protein analysis using the Biorad DC protein kit. To the remaining cell extracts in the viewplates, 215 ul of Microscint-20 were added and radioactive material was measured by liquid scintillation counting. Preliminary analyses carried out by TLC demonstrated that >95% of this radioactive material was nonmetabolized [$^3$H]anandamide, suggesting that our astrocytoma cell preparation contains no significant anandamide amidohyrolase activity.

Some of the inhibitors have been identified as competitive since they are recognized as substrates by the transporter and will undergo membrane translocation.

The $IC_{50}$ data in Table I provide the affinity data for ligand recognition by the anandamide transporter, but do not provide information on whether the ligands also may serve as substrates for the transporter. To investigate substrate translocation we used a representative set of radioactively labeled compounds. We tested four key analogs that

compete with anandamide for uptake: [$^3$H]*N*-(4-hydroxyphenyl)arachidonamide designated as AM404, and the materials designated AM1172, AM 1177 and AM1191 arachidonylglycerol. As shown in Figs1 and 2, all of the analogs are transported as rapidly and effectively as [$^3$H]anandamide at levels of 50 mM and 500 mM. These findings suggest that the anandamide transporter also may participate in the inactivation of 2-arachidonylglycerol, which was thought to be primarily mediated by enzymatic hydrolysis.

## Table I

| Structure | $IC_{50}$ |
|---|---|
| AM1191  | 0.8 |
| AM1177  | 5.0 |
| AM1172  | 2.0 |

Modifications of the hydrophobic fatty acid tail reveal unexpectedly distinct requirements for recognition and translocation of substrates by the anandamide transporter. Substrate recognition requires the presence of at least one cis double bond situated at the middle of the fatty acid chain, pointing to a preference for ligands in which the hydrophobic tail can fold in the middle and adopt a bent U-shaped conformation. Indeed, analogs with fully saturated chains or those incorporating trans double bonds do not interact significantly with the transporter. By contrast, substrate translocation requires a minimum of four cis nonconjugated double bonds, as ligands containing one, two, or three olefins are transported either very slowly or not at all. This finding suggests that for transmembrane transport to occur substrates must be capable of adopting a tightly folded conformation, one that is not energetically favorable for ligands containing an insufficient number of cis double bonds.

Molecular modeling studies of fatty acid ethanolamides differing in the degree of unsaturation of their hydrophobic carbon chains provides insight into these distinctive conformational requirements. Possible low-energy conformers of these molecules are significantly different. The presence of one or more nonconjugated cis double bonds in the middle of the chain or the use of a biphenyl group leads to the formation of a turn that brings in closer proximity the head and tail of the molecule. The shape of this turn is determined by the number and position of the cis double bonds. Conversely, the introduction of a central trans double bond yields a more extended chain conformation and hinders the ability of the molecule to undergo folding. Thus one of the low-energy conformers of anandamide displays a folded hairpin shape with the two halves of the molecule facing each other. The cis-triene analog may adopt an analogous conformation, though one that is wider than that of anandamide. The width of the turn increases

considerably in the cis-dienes and the two monoalkenes due to the marked increase in distance between the head group and tail of the molecule. In the corresponding trans alkene analog, the distance between the head and tail is much greater. It is important to point out that, whereas anandamide like arachidonic acid may adopt either a closed-hairpin or a U-shaped conformation depending on the properties of the surrounding milieu, the hairpin conformation may be thermodynamically unfavorable to fatty acid ethanolamides containing only one or two double bonds.

A plausible interpretation of our results is that recognition and translocation of substrates by the anandamide transporter are governed by distinct conformational preferences. Although the initial recognition step may require that substrates assume a bent U-shaped conformation of variable width, the subsequent step of translocation across the cell membrane may impose a more tightly folded hairpin conformation.

A "therapeutically effective amount" of a compound, as used herein, is the quantity of a compound which, when administered to an individual or animal, results in a sufficiently high level of anandamide in the individual or animal to cause a discernable increase or decrease in a cellular activity affected or controlled by cannabinoid receptors. For example, anandamide can stimulate receptor-mediated signal transduction that leads to the inhibition of forskolin-stimulated adenylate cyclase (Vogel *et al., J. Neurochem.* 60:352 (1993)). Anandamide also causes partial inhibition of N-type calcium currents via a pertussis toxin-sensitive G protein pathway, independently of cAMP metabolism (Mackie *et al., Mol. Pharmacol.* 47:711 (1993)).

A "therapeutically effective amount" of an anandamide inhibitor can also be an amount which results in a sufficiently high level of anandamide in an individual or animal to cause a physiological effect resulting from stimulation of cannabinoid receptors. Physiological

effects which result from cannabinoid receptor stimulation include analgesia, decreased nausea resulting from chemotherapy, sedation and increased appetite. Other physiological functions include relieving intraocular pressure in glaucoma patients and suppression of the immune system. Typically, a "therapeutically effective amount" of the compound ranges from about 10 mg/day to about 1,000 mg/day.

As used herein, an "individual" refers to a human. An "animal" refers to veterinary animals, such as dogs, cats, horses, and the like, and farm animals, such as cows, pigs, guinea pigs and the like.

The compounds of the present invention can be administered by a variety of known methods, including orally, rectally, or by parenteral routes (e.g., intramuscular, intravenous, subcutaneous, nasal or topical). The form in which the compounds are administered will be determined by the route of administration. Such forms include, but are not limited to, capsular and tablet formulations (for oral and rectal administration), liquid formulations (for oral, intravenous, intramuscular or subcutaneous administration and slow releasing microcarriers (for rectal, intramuscular or intravenous administration). The formulations can also contain a physiologically acceptable vehicle and optional adjuvants, flavorings, colorants and preservatives. Suitable physiologically acceptable vehicles may include saline, sterile water, Ringer's solution, and isotonic sodium chloride solutions. The specific dosage level of active ingredient will depend upon a number of factors, including, for example, biological activity of the particular preparation, age, body weight, sex and general health of the individual being treated.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to specific embodiments of the invetnion described specifically herein. Such equivalents are intended to be encompassed in the scope of the invention.

**Claims:**

1. A method of inhibiting transport of anandamide in an individual or animal comprising administering to the individual or animal a therapeutically effective amount of a compound represented by the following structural formula:

X - Y - Z

and physiologically acceptable salts thereof, wherein:

X is a member selected from the group consisting of a hydrophobic aliphatic hydrocarbon chain containing from about 4 to about 30 carbon atoms and having one or more nonconjugated cis double bonds in the middle portion of the chain with a terminal radical selected from the group consisting of hydrogen, aryl and aryl substituted with a member selected from the group consisting of hydroxy, halogen, $-NO_2$, $-NH_2$, $-CH_3$, $-OCH_3$ and $-SCH_3$, or biphenyl or biphenyl having a terminal straight or branched alky group of about 1 to about 10 carbon atoms;

Y is selected from the group consisting of hydrogen, -NH-C(O)-, -NH-, -NH-C(O)-NH-, -NH-C(O)O-, -C(O)-NH-, -O-C(O)-, -O- and -S-; and

Z is selected from the group consisting of hydrogen, aryl, alkyl aryl, halogen substituted alkyl aryl, cyclic glycerols and substituted cyclic glycerols.

2. The method of claim 1 wherein the radicals on the substituted cyclic glycerol are selected from the group consisting of lower alkyl of about 1 to about 5 carbon atoms, aryl and substituted aryl.

3. The method of claim 1 wherein Y is a carbonyl amine radical.

4. The method of claim 1 wherein X is a biphenyl having a terminal alkyl group.

5. The method of claim 1 wherein X is an aliphatic hydrocarbon chain having two or more nonconjugated double bonds.

6. The method of claim 1 wherein X is an aliphatic hydrocarbon chain having at least four nonconjugated double bonds.

7. The method of claim 1 wherein Z is a hydroxy substituted aryl group.

8. A compound represented by the following structural formula:

X - Y - Z

and physiologically acceptable salts thereof, wherein:

X is a member selected from the group consisting of a hydrophobic aliphatic hydrocarbon chain containing from about 4 to about 30 carbon atoms and having one or more nonconjugated cis double bonds in the middle portion of the chain with a terminal radical selected from the group consisting of hydrogen, aryl and aryl substituted with a member selected from the group consisting of hydroxy, halogen, $-NO_2$, $-NH_2$, $-CH_3$, $-OCH_3$ and $-SCH_3$, or biphenyl or biphenyl having a terminal straight or branched alky group of about 1 to about 10 carbon atoms;

Y is selected from the group consisting of hydrogen, -NH-C(O)-, -NH-, -NH-C(O)-NH-, -NH-C(O)O-, -C(O)-NH-, -O-C(O)-, -O- and -S-; and

Z is selected from the group consisting of hydrogen, aryl, alkyl aryl, halogen substituted alkyl aryl, cyclic glycerols and substituted cyclic glycerols.

9. The compound of claim 8 wherein the radicals on the substituted cyclic glycerol are selected from the group consisting of lower alkyl of about 1 to about 5 carbon atoms, aryl and substituted aryl

10. The compound of claim 8 wherein Y is a carbonyl amine radical.

11. The compound of claim 8 wherein X is a biphenyl having a terminal alkyl group.

12. The compound of claim 8 wherein X is an aliphatic hydrocarbon chain having two or more nonconjugated double bonds.

13. The compound of claim 8 wherein X is an aliphatic hydrocarbon chain having at least four nonconjugated double bonds.

14. The compound of claim 8 wherein Z is a hydroxy substituted aryl group.

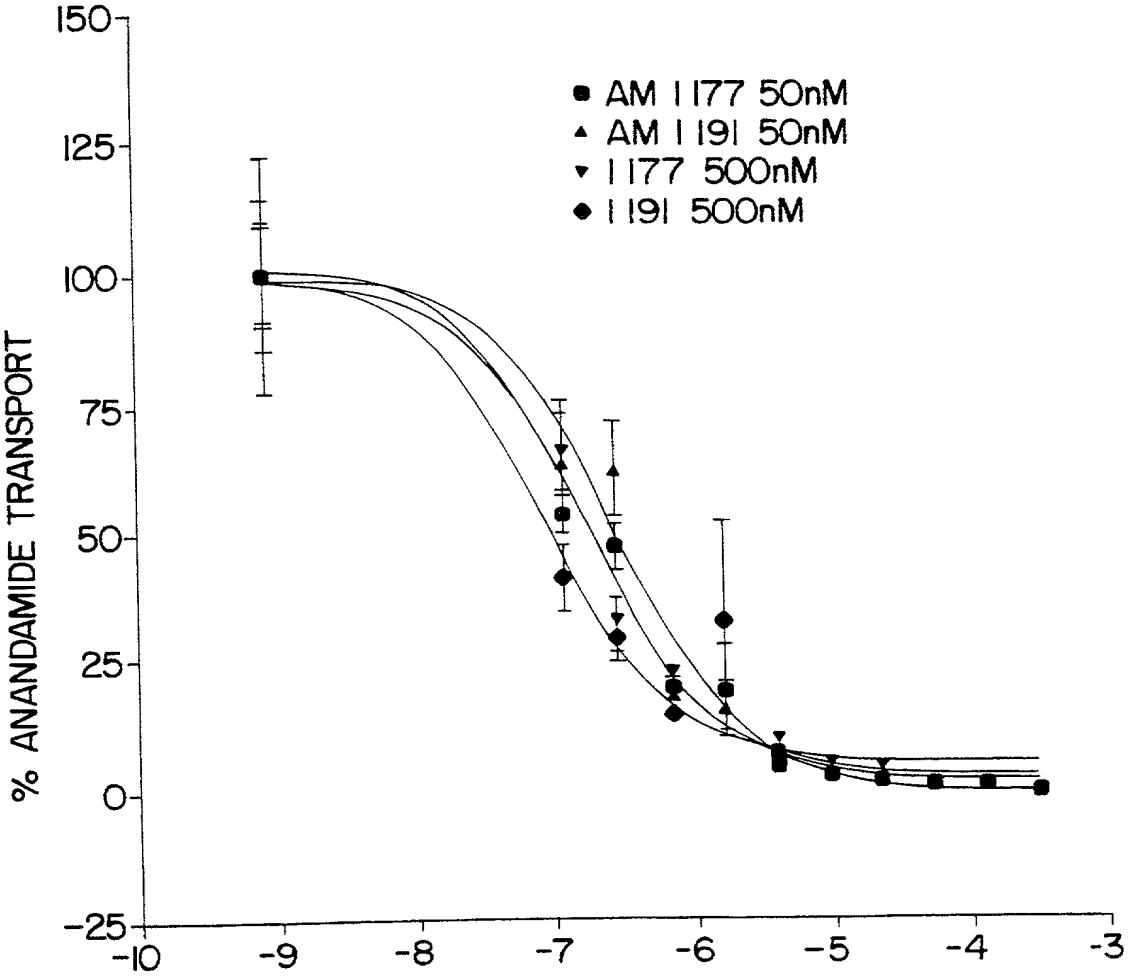

150
125
100
75
50
25
0
-25
-10
-9
-8
-7
-6
-5
-4
-3
% ANANDAMIDE TRANSPORT
AM 1177 50nM
AM 1191 50nM
1177 500nM
1191 500nM

FIG. 1

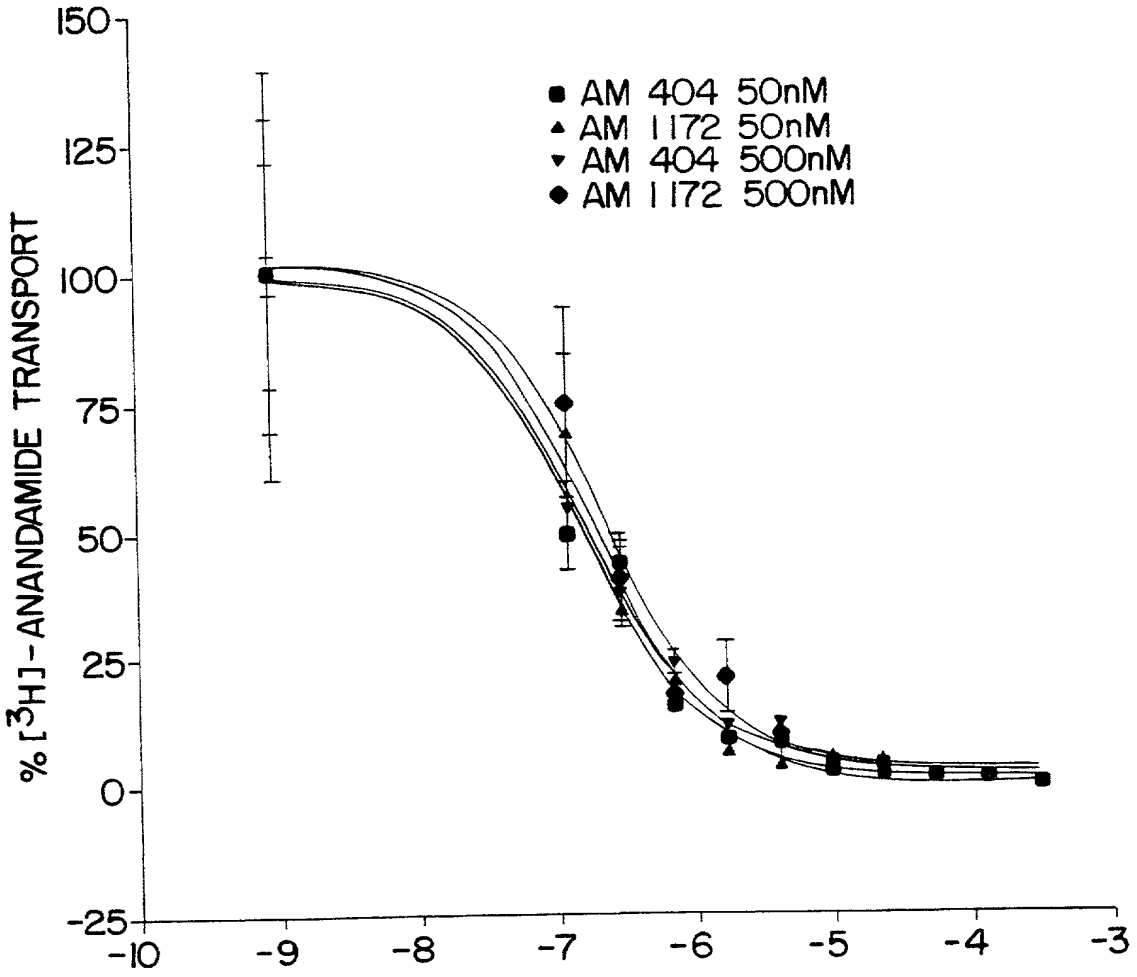

150
125
100
75
50
25
0
-25
-10
-9
-8
-7
-6
-5
-4
-3
% [3H]-ANANDAMIDE TRANSPORT
AM 404 50nM
AM 1172 50nM
AM 404 500nM
AM 1172 500nM

FIG.2

Type a plus sign [ + ] inside this box → [+]

| | | | |
|---|---|---|---|
| 0010/PTO<br>Rev. 6/95 | U.S. Department of Commerce<br>Patent and Trademark Office | Attorney Docket | **UCON/150/PC/US** |
| | | First Named Inventor | Alexandros Makriyannis |
| | | COMPLETE IF KNOWN | |
| **DECLARATION** | | Application Number | |
| ☒ Declaration Submitted With Initial Filing | ☐ Declaration Submitted After Initial Filing | Filing Date | |
| | | Group Art Unit | |
| | | Examiner Name | |



As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

INHIBITORS OF THE ANANDAMIDE TRANSPORTER AS ANALGESIC AGENTS

(Title of the Invention)

the specification of which

☐ is attached hereto.

OR

☒ Was filed on (MM/DD/YYYY) 06/09/1999 as United States Application or PCT International Application Number PCT/US99/12900 and was amended on (MM/DD/YYYY) _____ (if applicable).

I hereby state that I have reviewed and understood the contents of the above-identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37 Codes of Federal Regulations, §1.56.

I hereby claim foreign priority under Title 35, United States Code § 119 (a)-(d) or § 365 (b) of any foreign application(s) for patent or inventor's certificate, or § 365 (a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

| Prior Foreign Application Numbers | Country | Foreign Filing Date (MM/DD/YYYY) | Priority Not Claimed | Copy Attached Yes | Copy Attached No |
|---|---|---|---|---|---|
| NONE | | | ☐ | ☐ | ☐ |
| | | | ☐ | ☐ | ☐ |
| | | | ☐ | ☐ | ☐ |
| | | | ☐ | ☐ | ☐ |

☐ Additional Foreign Application Numbers Are Listed On A Supplemental Priority Sheet Attached Hereto:

☒ I hereby claim the benefit under Title 35, United States Code § 119 (e) of any United States provisional application(s) listed below:

| Application Number(s) | Filing Date [MM/DD/YY] | |
|---|---|---|
| 60/088,568 | 06/09/1998 | ☐ Additional Provisional Application Numbers Are Listed On A Supplemental Priority Sheet Attached Hereto. |

| DECLARATION | Page 2 |
|---|---|

I hereby claim the benefit under Title 35, United States Code §120 of any United States application(s), or §365(c) of any PCT International application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Title Code of Federal Regulations §1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

| U.S. Parent Application Number | PCT Parent Number | Parent Filing Date (MM/DD/YYYY) | Parent Patent Number (if applicable) |
|---|---|---|---|
| NONE | | | |



☐ Additional U.S. Or PCT International Application Numbers Are Listed On A Supplementary Priority Sheet Attached Hereto:

As a named inventor, I hereby appoint in the registered practitioners associated with the Customer Number provided below to prosecute this application and to transact all business in the Patent and Trademark Office therewith, and direct that all correspondence be addressed to that Customer Number:

Firm Name: **Alix, Yale & Ristas, LLP** Customer Number: **002543**

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name Of Sole Or First Inventor ☐ A Petition Has Been Filed For This Unsigned Inventor

| Given Name | Alexandros | Middle Initial | | Family Name | MAKRIYANNIS | Suffix | |
|---|---|---|---|---|---|---|---|
| Inventor's Signature | x Alexandros Makriyannis | | | | Date | 12-8-00 | |
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| City | Willimantic | State | CT | Zip | 06226 | Country | U.S.A. |
| Applicant Authority | | | | | | | |

Name Of Additional Joint Inventor, If Any: ☐ A Petition Has Been Filed For This Unsigned Inventor

| Given Name | Andreas | Middle Initial | | Family Name | GOUTOPOULOS | Suffix | |
|---|---|---|---|---|---|---|---|
| Inventor's Signature | x Andreas Goutopoulos | | | | Date | 12/12/2000 | |
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| City | BOSTON | State | MA | Zip | 02116 | Country | U.S.A. |
| Applicant Authority | | | | | | | |

☐ Additional Inventors Are Being Named On Supplemental Sheet(s) Attached Hereto.